

Patent Application
Attn. Docket No. MUR-003

"Genetic Suppression and Replacement"

Reference to Related Applications

5 This application was filed under 35 U.S.C. §371 for,
and claims priority to, PCT/GB97/00929, filed April 2,
1997, which claims priority to GB9606961.2, filed April 2,
1996, the disclosures of which are incorporated by
reference herein.

Field of the Invention

10 The present invention relates to a strategy for
suppressing a gene. In particular the invention relates
to suppression of a mutated gene that gives rise to a
dominant or deleterious effect, either monogenically or
polygenically. The present invention relates to a
strategy for suppressing a gene. In particular the
invention relates to suppression of mutated genes which
15 give rise
to a dominant or deleterious effect, either monogenically
or polygenically. The invention relates to a strategy for
suppressing a gene or disease allele using methods which
do not target the disease allele specifically but instead
20 can be targeted towards a broad range of sequences in a
particular gene. A particular embodiment of the invention
is the use of suppression strategies to target either the
disease or normal alleles alone or to target both disease
and normal alleles. A further embodiment of the invention

is the use of the wobble hypothesis to enable continued expression of a replacement normal or beneficial gene (a gene modified from the wild type such that it provides an additional beneficial effect(s)). The replacement gene will have nucleotide changes from the endogenous wild type gene but will code for identical amino acids as the wild type gene. The strategy circumvents the need for a specific therapy for every mutation within a given gene. In addition the invention allows greater flexibility in choice of target sequence for suppression of a disease allele.

The invention also relates to a medicament or medicaments for use in suppressing a deleterious allele which is present in a genome of one or more individuals or animals.

Generally the present invention will be useful where the gene, which is naturally present in the genome of a patient, contributes to a disease state. Generally, one allele of the gene in question will be mutated, that is, will possess alterations in its nucleotide sequence that affects the function or level of the gene product. For example, the alteration may result in an altered protein product from the wild type gene or altered control of transcription and processing. Inheritance or somatic acquisition of such a mutation can give rise to a disease

~~phenotype or can predispose an individual to a disease phenotype. However the gene of interest could also be of wild type phenotype, but~~

~~contribute to a disease state in another way such that the suppression of the gene would alleviate or improve the disease state or improve the effectiveness of an administered therapeutic compound.~~

~~Generally, suppression effectors such as nucleic acids antisense or sense, ribozymes, peptide nucleic acids (PNAs), triple helix forming oligonucleotides, peptides and /or antibodies directed to sequences in a gene, in transcripts or in protein, can be employed in the invention to achieve gene suppression.~~

~~BACKGROUND~~ Background of the Invention

Studies of degenerative hereditary ocular conditions, including Retinitis Pigmentosa (RP) and various macular dystrophies, have resulted in a substantial elucidation of the molecular basis of these debilitating human retinal degenerations. Applying the approach of genetic linkage, x-linked RP (xLRP) genes have been localised to the short arm of the X chromosome (Ott et al. 1990). S

subsequently, the gene involved in one form of xLRP has been was identified. Various genes involved in autosomal dominant forms of RP (adRP) have been localised. The first of these mapped ~~on~~ to 3q, close to the gene encoding the rod photoreceptor protein rhodopsin (McWilliam et al. 1989; Dryja et al. 1990).

Similarly, an adRP gene was placed on 6p close to the gene encoding the photoreceptor protein peripherin (Farrar et al. 1991a,b; Kajiwara et al. 1991).

Other adRP genes have been mapped to discrete chromosomal locations; however the disease genes as yet remain uncharacterised. As in xLRP and adRP, various genes involved in autosomal recessive RP (arRP) have been localised and in some cases molecular defects characterised (Humphries et al. 1992; Farrar et al. 1993; Van Soest et al. 1994). Similarly, a number of genes involved in macular dystrophies have been mapped (Mansergh et al. 1995). Genetic linkage, together with

techniques for mutational screening of candidate genes, enabled identification of causative dominant mutations in the genes encoding rhodopsin and peripherin. Globally, about 100 rhodopsin mutations have been found in patients with RP or congenital stationary night blindness. Similarly, approximately 40 mutations have

been characterised in the peripherin gene in patients with RP or macular dystrophies. Knowledge of the molecular aetiology of these retinopathies has

stimulated the generation of animal models and the exploration of methods of therapeutic intervention (Farrar et al. 1995; Humphries et al. 1997). —

Similar to RP, osteogenesis imperfecta (OI) is an autosomal dominantly inherited human disorder whose molecular pathogenesis is extremely genetically heterogeneous. OI is often referred to as 'brittle bone disease', although additional symptoms including hearing loss, growth deficiency, bruising, loose joints, blue sclerae and dentinogenesis imperfecta are frequently observed (McKusick, 1972). Mutations in the genes encoding the two type I collagen chains (collagen 1A1 and 1A2) comprising the type I collagen heterodimer have been implicated in OI. Indeed hundreds of dominantly acting mutations have been identified in OI patients in these two genes, many of which are single point mutations, although a number of insertion and deletion mutations have been found (Willing et al. 1993; Zhuang et al. 1996). Similarly mutations in these genes have also been implicated in Ehlers-Danlos and Marfan syndromes (Dalglish et al. 1986; Phillips et al. 1990; D'Alessio et al. 1991; Vasan NS et al. 1991).

Generally, gene therapies utilising viral and non-viral delivery systems have been used to treat inherited disorders, cancers and infectious diseases. However, many studies have focused on recessively inherited disorders, the rationale being that introduction and expression of the wild type gene may be sufficient to prevent/ameliorate the disease phenotype. In contrast gene therapy for dominant disorders ~~will~~ requires suppression of the dominant disease allele. Notably many of the characterised mutations causing inherited diseases such as RP or OI are inherited in an autosomal dominant fashion. Indeed there are over 1,000 autosomal dominantly inherited disorders in man. In addition, there are many polygenic disorders due to co-inheritance of a number of genetic components

which together give rise to the disease state. Effective gene therapies for dominant or polygenic diseases may be targeted to the primary defect and in this case may require suppression of the disease allele while in many cases still maintaining the function of the normal allele. This ~~will be~~ is particularly relevant where disease pathology is due to a gain of function mutation rather than due to reduced levels of wild type protein.

Alternatively, suppression therapies may be targeted to secondary effects associated with the disease pathology,

~~for one example, is~~ programmed cell death (apoptosis),
which has been observed in many inherited disorders.

_____ Strategies to differentiate between normal and
5 disease alleles and to selectively switch off the disease
allele using suppression effectors such as ~~inter alia~~
antisense DNA/RNA, PNAs, ribozymes, or triple helix
forming DNA, targeted towards the disease mutation may be
difficult in many cases -- frequently disease and normal
10 alleles differ by only a single nucleotide. A further
difficulty inhibiting development of gene therapies is the
heterogeneous nature of some dominant disorders -- many
different mutations in the same gene give rise to a
similar disease phenotype. Development of specific gene
15 therapies for each of these may be prohibitive in terms of
cost. ~~To circumvent difficulties associated with~~
~~specifically targeting the disease mutation and with the~~
~~genetic heterogeneity present in inherited disorders, a~~
~~novel strategy for gene suppression and gene replacement~~
20 ~~exploiting the degeneracy of the genetic code, thereby~~
~~allowing flexibility in choice of target sequence for~~
~~suppression and providing a means of gene suppression~~
~~which is independent of the disease mutation, is described~~
~~in the invention.~~

25

~~PRIOR ART~~

____ Suppression effectors have been used previously to achieve specific suppression of gene expression.

5 Antisense DNA and RNA has been used to inhibit gene expression in many instances. Modifications, such as phosphorothioates, have been made to oligonucleotides to increase resistance to nuclease degradation, binding affinity and uptake (Cazenave et al. 1989; Sun et al. 10 1989; McKay et al. 1996; Wei et al. 1996). In some instances, using antisense and ribozyme suppression strategies has led to reversal of a tumor phenotype by reducing expression of a gene product or by cleaving a mutant transcript at the site of the mutation (Carter and 15 Lemoine 1993; Lange et al. 1993; Valera et al. 1994; Dosaka--Akita et al. 1995; Feng et al. 1995; Quattrone et al. 1995; Ohta et al. 1996). For example, neoplastic reversion was obtained using a ribozyme targeted to an H-- ras mutation in bladder carcinoma cells (Feng et al. 20 1995). Ribozymes have also been proposed as a means of both inhibiting gene expression of a mutant gene and of correcting the mutant by targeted trans--splicing (Sullenger and Cech 1994; Jones et al. 1996). Ribozymes can be designed to elicit autocatalytic cleavage of RNA 25 targets; however, the inhibitory effect of some ribozymes may be due in part to an antisense effect due to the

antisense sequences flanking the catalytic core ~~which~~that specify the target site (Ellis and Rodgers 1993; Jankowsky and Schwenzer 1996). Ribozyme activity may be augmented by the use of, for example, non--specific nucleic acid binding proteins or facilitator oligonucleotides (Herschlag et al. 1994; Jankowsky and Schwenzer 1996). Multitarget ribozymes (connected or shotgun) have been suggested as a means of improving efficiency of ribozymes for gene suppression (Ohkawa et al. 1993). Triple helix approaches have also been investigated for sequence-specific gene suppression -- triplex forming oligonucleotides have been found in some cases to bind in a sequence--specific manner (Postel et al. 1991; Duval--Valentin et al. 1992; Hardenbol and Van Dyke 1996; Porumb et al. 1996). Similarly, peptide nucleic acids have been shown to inhibit gene expression (Hanvey et al. 1992; Knudson and Nielsen 1996; Taylor et al. 1997). Minor groove binding polyamides can bind in a sequence--specific manner to DNA targets and hence may represent useful small molecules for future suppression at the DNA level (Trauger et al. 1996). In addition, suppression has been ~~obtained~~ achieved by interference at the protein level using dominant negative mutant peptides and antibodies (Herskowitz 1987; Rimsky et al. 1989; Wright et al. 1989). In some cases suppression strategies have lead to a reduction in RNA levels without a concomitant reduction in proteins, whereas in others, reductions in RNA have been mirrored by reductions in protein.

~~There is now an armament with which to obtain gene suppression. This, in conjunction with a better understanding of the molecular etiology of disease,~~

5 ~~results in an ever increasing number of disease targets for therapies based on suppression. In many cases, complete suppression of gene expression has been~~

~~difficult to achieve. Possibly a combined approach using a number of suppression effectors may aid in this. For~~

10 ~~some disorders it may be necessary to block~~

~~expression of a disease allele completely to prevent disease symptoms whereas for others low levels of mutant protein may be tolerated. In parallel with an~~

~~increased knowledge of the molecular defects causing~~

15 ~~disease has been the realisation that many disorders are genetically heterogeneous. Examples in which~~

~~multiple genes and/or multiple mutations within a gene can give rise to a similar disease phenotype include~~

~~osteogenesis imperfecta, familial hypercholesterolemia,~~

20 ~~retinitis pigmentosa, and many others. The utility of the degeneracy of the genetic code (wobble hypothesis) to enable suppression of one or both alleles of a gene and the introduction of a replacement gene such that~~

~~it escapes suppression has been exploited in the~~
invention. Summary of the Invention

To circumvent difficulties associated with
specifically targeting a disease mutation and with the
5 genetic heterogeneity present in inherited disorders, a
novel strategy for gene suppression and gene replacement
exploiting the degeneracy of the genetic code is
described. The invention allows flexibility in choice of
target sequence for suppression and provides a means of
10 gene suppression that is independent of the disease
mutation.

In summary, the invention involves gene suppression
of disease and normal alleles targeting coding sequences
in a gene and, when necessary, gene replacement such that
15 the replacement gene cannot be suppressed. Replacement
genes are modified at third base positions (wobble
positions) so that they code for the correct amino acids
but are protected completely or partially from
suppression. The same suppression and replacement steps
20 can be used for many disease mutations in a given gene.
Suppression and replacement can be undertaken in
conjunction with each other or separately.

The invention relates to a strategy for suppressing a
25 gene or disease allele using methods that do not target
the disease allele specifically but instead can be

targeted towards a broad range of sequences in a particular gene. A particular embodiment of the invention is the use of suppression strategies to target either the disease or normal alleles alone or to target both disease and normal alleles. A further embodiment of the invention is the use of the wobble hypothesis to enable continued expression of a replacement normal or beneficial gene (a gene modified from the wild type such that it provides an additional beneficial effect(s)). The replacement gene will have nucleotide changes from the endogenous wild type gene but will code for identical amino acids as the wild type gene. The strategy circumvents the need for a specific therapy for every mutation within a given gene. In addition, the invention allows greater flexibility in choice of target sequence for suppression of a disease allele.

The invention also relates to a medicament or medicaments for use in suppressing a deleterious allele that is present in a genome of one or more individuals or animals.

Generally, the present invention will be useful where the gene, which is naturally present in the genome of a patient, contributes to a disease state. Generally, one allele of the gene in question will be mutated, that is, will possess alterations in its nucleotide sequence that affects the function or level of the gene product. For example, the alteration may result in an altered protein

product from the wild type gene or altered control of transcription and processing. Inheritance or somatic acquisition of such a mutation can give rise to a disease phenotype or can predispose an individual to a disease phenotype. However the gene of interest could also be of wild type phenotype, but contribute to a disease state in another way such that the suppression of the gene would alleviate or improve the disease state or improve the effectiveness of an administered therapeutic compound.

Generally, suppression effectors such as nucleic acids - antisense or sense, ribozymes, peptide nucleic acids (PNAs), triple helix forming oligonucleotides, peptides and /or antibodies directed to sequences in a gene, in transcripts or in protein, can be employed in the invention to achieve gene suppression.

Detailed Description of the Invention

The invention addresses shortcomings of the prior art by providing a novel approach to the design of suppression effectors directed to target alleles of a

gene carrying a deleterious mutation. Suppression of every mutation giving rise to a disease phenotype may be costly and problematic. Disease mutations are

often single nucleotide changes. As a result

differentiating between the disease and normal alleles may

be difficult. Some suppression effectors require specific sequence targets, for example, hammerhead ribozymes cleave at NUX sites and hence may not be able to target many mutations. Notably, the wide spectrum of mutations
5 observed in many diseases adds additional complexity to the development of therapeutic strategies for such disorders -- some mutations may occur only once in a single patient. A further problem associated with
_suppression is the high level of homology present in
10 coding sequences between members of some gene families. This can limit the range of target sites for suppression ~~which~~ that will enable specific suppression of a single member of such a gene family.

15 _____The present invention circumvents shortcomings in the prior art by utilising the degeneracy of the genetic code. In the invention suppression effectors are
designed specifically to sequences in coding regions of genes or in gene products. Typically, one allele of the
20 gene contains a mutation with a deleterious effect. Suppression targeted to coding sequences provides greater flexibility in choice of target sequence for suppression in contrast to suppression directed towards single disease
mutations. Additionally, the invention provides for the
25 introduction of a replacement gene with modified sequences such that the replacement gene is protected from

suppression. The replacement gene is modified at third
base wobble positions and hence provides the wild type
gene product. Notably, the invention has the advantage
that the same suppression strategy could be used to
5 suppress, in principle, many

mutations in a gene. This is particularly relevant when
large numbers of mutations within a single gene cause
disease pathology. The replacement gene provides (when
necessary) expression of the normal protein product when
10 required to ameliorate pathology associated with reduced
levels of wild type protein. The same replacement gene
could in principle be used in conjunction with the
suppression of many different disease mutations within a
given gene. Target

15 sequences may be found in any part of the coding sequence.
Suppression in coding sequence holds the advantage that
such sequences are present in both
precursor and mature RNAs, thereby enabling suppressors to
target all forms of RNA.

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There is now an armament with which to obtain gene
suppression. This, in conjunction with a better
understanding of the molecular aetiology of disease,
results in an ever increasing number of disease targets
25 for therapies based on suppression. In many cases,
complete suppression of gene expression has been difficult

to achieve. Possibly a combined approach using a number of suppression effectors may aid in this. For some disorders it may be necessary to block expression of a disease allele completely to prevent disease symptoms whereas for others low levels of mutant protein may be tolerated. In parallel with an increased knowledge of the molecular defects causing disease has been the realisation that many disorders are genetically heterogeneous. Examples in which multiple genes and/or multiple mutations within a gene can give rise to a similar disease phenotype include osteogenesis imperfecta, familial hypercholesterolemia, retinitis pigmentosa, and many others. The utility of the degeneracy of the genetic code (wobble hypothesis) to enable suppression of one or both alleles of a gene and the introduction of a replacement gene such that it escapes suppression has been exploited in the invention.

~~In summary the invention involves gene suppression of disease and normal alleles targeting coding sequences in a gene and when necessary gene replacement such that the replacement gene cannot be suppressed. Replacement genes are modified at third base positions (wobble positions) so that they code for the correct amino acids but are protected completely or partially from suppression. The same suppression and replacement steps can be used for many disease mutations in a given gene.~~

~~Suppression and replacement can be undertaken in
conjunction with each other or separately.~~

5 ~~DESCRIPTION OF INVENTION~~

____ According to the present invention there is provided
a strategy for suppressing expression of an endogenous
gene with a deleterious mutation, wherein said strategy
10 comprises providing suppression effectors such as
antisense nucleic acids able to bind to sequences of a
gene to be suppressed, to prevent the functional
expression thereof.

15 ____ Generally the term suppression effectors means
nucleic acids, peptide nucleic acids (PNAs), peptides,
antibodies or modified forms of these used to silence or
reduce gene expression in a sequence specific manner.

20 ____ Suppression effectors, such as antisense nucleic
acids can be DNA or RNA, can typically be directed to
coding sequence; however suppression effectors can be
targeted to coding sequence and can also be

~~targetted~~targeted to 5' and/or 3' untranslated regions and/or introns and/or control regions and/or sequences adjacent to a gene or to any combination of such regions of a gene. Antisense nucleic acids including both coding and non--coding sequence can be used if required to help to optimise suppression. Binding of the suppression effector(s) prevents or lowers functional expression of the endogenous gene.

10 | _____ Generally the term 'functional expression' means the expression of a gene product able to function in a manner equivalent to, or better than, a wild type product. In the case of a mutant gene or predisposing gene 'functional expression' means the expression of a gene product whose
15 | presence gives rise to a deleterious effect or predisposes to a deleterious effect. By deleterious effect is meant giving rise to or predisposing to disease pathology or altering the effect(s) and/or efficiency of an administered compound.

20 | _____ In a particular embodiment of the invention the strategy further employs ribozymes ~~which~~that can be designed to elicit cleavage of target RNAs. The strategy further employs nucleotides ~~which~~that form triple helix
25 | DNA. The strategy can employ peptide nucleic acids for suppression. Nucleic acids for antisense, ribozymes,

triple helix forming DNA and peptide nucleic acids may be modified to increase stability, binding efficiencies and uptake ~~(see prior art)~~. Nucleic acids can be incorporated into a vector. Vectors include naked DNA, DNA plasmid
5 vectors, RNA or DNA virus vectors, lipids, polymers or other derivatives and compounds to aid gene delivery and expression.

10 The invention further provides the use of antisense nucleotides, ribozymes, PNAs, triple helix nucleotides, or other suppression effectors alone or in a vector or vectors, wherein the nucleic acids are able to bind specifically or partially specifically to coding sequences of a gene to prevent or reduce the functional expression
15 thereof, in the preparation of a medicament for the treatment of an autosomal dominant or polygenic disease or to increase the utility and/or action of an administered compound.

20 In a further embodiment of the invention, target sequences for suppression can include non-coding sequences of the gene.

____ According to the present invention there is provided
a strategy for suppressing specifically or partially
specifically an endogenous gene and (if required)

introducing a replacement gene, said strategy comprising
5 the steps of:

1. providing nucleic acids or other suppression
effectors able to bind to an endogenous gene, gene
transcript or gene product to be suppressed and
- 10 2. providing genomic DNA or cDNA (complete or partial)
encoding a replacement gene- wherein the nucleic
acids are unable to bind to equivalent regions in the
genomic DNA or cDNA to prevent expression of the
15 replacement gene. The replacement nucleic acids will
not be recognised by suppression nucleic acids or
will be recognised less effectively than the
endogenous gene. The coding sequence of replacement
nucleic acids can be altered to prevent or reduce
20 efficiency of suppression. Replacement nucleic acids
have modifications in one or more third base (wobble)
positions such that replacement nucleic acids still
code for the wild type or equivalent amino acids.

_____ In a particular embodiment of the invention there is provided a strategy for gene suppression targeted to coding sequences of the gene to be suppressed.

Suppression will be specific or partially specific to one allele, for example, to the allele carrying a deleterious mutation. Suppressors are targeted to

coding regions of a gene or to a combination of coding and non--coding regions of a gene. Suppressors can be targeted to a characteristic of one allele of a gene

10 such that suppression is specific or partially specific to one allele of a gene (PCT/GB97/00574). The invention further provides for use of replacement nucleic acids with altered coding sequences such that replacement nucleic acids will not be recognised (or will be recognised less
15 effectively) by suppression nucleic acids ~~which~~ that are targeted specifically or partially specifically to one allele of the gene to be suppressed. Replacement nucleic acids provide the wild type gene product, an equivalent gene product or an improved gene product but are protected
20 completely or partially from suppression effectors targeted to coding
_sequences.

_____ In a further embodiment of the invention, replacement nucleic acids are provided such that replacement nucleic acids will not be recognised by naturally

occurring suppressors found to inhibit or reduce gene
5 expression in one or more individuals, animals or plants.

The invention provides for use of replacement
nucleic acids ~~which~~ that have altered sequences targeted
by suppressors of the gene such that suppression by
naturally occurring suppressors is completely or
10 partially prevented.

_____ In an additional embodiment of the invention, there is
provided replacement nucleic acids with altered nucleotide
sequence in coding regions such that
15 replacement nucleic acids code for a product with one or
more altered amino acids. Replacement nucleic acids
provide a gene product ~~which~~ that is equivalent to
or improved compared with the naturally occurring
endogenous wild type gene product.

20 _____ In an additional embodiment of the invention there is
provided a strategy to suppress a gene where the gene

transcript or gene product interferes with the action of
an administered compound.

____ The invention further provides the use of a vector or
5 vectors containing suppression effectors in the form of
nucleic acids, said nucleic acids being directed towards
coding sequences or combinations of coding and non--coding
sequences of the target gene and vector(s) containing
genomic DNA or cDNA encoding a replacement gene sequence
10 to which nucleic acids for suppression are unable to bind
(or bind less efficiently), in the preparation of a
combined medicament for the treatment of an autosomal
dominant or polygenic disease. Nucleic acids for
suppression or replacement gene nucleic acids may be
15 provided in the same vector or in separate vectors.
Nucleic acids for suppression or replacement gene nucleic
acids may be provided as a combination of nucleic acids
alone or in vectors.

20 The invention further provides a method of treatment for a
disease caused by an endogenous mutant gene, said method
comprising sequential or concomitant introduction of

(a) nucleic acids to the coding regions of a gene to be
25 suppressed and/or nucleic acids to coding regions and

any combination of 5' and/or 3' untranslated regions,
intronic regions, control regions or regions adjacent
to a gene to be suppressed

- 5 (b) replacement nucleic acids with sequences ~~which~~ that
allow the replacement gene to be expressed.

____ The nucleic acid for gene suppression can be
administered before, after or at the same time as the
10 replacement gene is administered.

____ The invention further provides a kit for use in the
treatment of a disease caused by a deleterious mutation in
a gene, the kit comprising nucleic acids for suppression
15 able to bind to the gene to be suppressed and if required
a replacement nucleic acid to replace the mutant gene
having sequence ~~which~~ that allows it to be expressed and
completely or partially escape suppression.

20 ____ Nucleotides can be administered as naked DNA or RNA.
Nucleotides can be delivered in vectors. Naked nucleic
acids or nucleic acids in vectors can be delivered with
lipids or other derivatives which aid gene delivery.
Nucleotides may be modified to render them more stable,

for example, resistant to cellular nucleases while still supporting RNase_H mediated degradation of RNA or with increased binding efficiencies ~~(see prior art)~~.

Antibodies or peptides can be generated to target the
5 protein product from the gene to be suppressed.

_____The strategy described herein has applications for alleviating autosomal dominant diseases. Complete silencing of a disease allele may be difficult to

10 achieve using antisense, PNA, ribozyme and triple helix approaches or any combination of gene silencing approaches. However small quantities of mutant product may be tolerated in some autosomal dominant disorders. In others a significant reduction in the proportion of mutant
15 to normal product may result in an amelioration of disease symptoms. Hence, this invention may be applied to any autosomal dominantly or polygenically inherited disease in man where the molecular basis of the disease has been established or is partially understood. This strategy
20 ~~will enables~~ the same therapy to be used to treat a range of different disease mutations within the same gene. The development of such approaches ~~will be~~ is important to future therapies for autosomal dominant and polygenic diseases, the key to a general strategy being that it
25 circumvents the need for a specific therapy for every mutation causing or predisposing to a disease. This is particularly relevant in some disorders, for example,

rhodopsin linked autosomal dominant RP, in which to date about one hundred different mutations in the rhodopsin gene have been observed in adRP patients. Likewise, hundreds of mutations have been identified in the human type I Collagen 1A1 and 1A2 genes in autosomal dominant osteogenesis imperfecta. Costs of developing therapies for each mutation are prohibitive at present. Inventions such as this using a general approach for therapy will be required. General approaches may be targeted to the primary defect, as is the case with this invention, or to secondary effects such as apoptosis.

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This invention may be applied in gene therapy approaches for biologically important polygenic disorders affecting large proportions of the world's populations such as age related macular degeneration, glaucoma, manic depression, cancers having a familial component and indeed many others. Polygenic diseases require inheritance of more than one mutation (component) to give rise to the disease state. Notably an amelioration in disease symptoms may require reduction in the presence of only one of these components, that is, suppression of one genotype which, together with others leads to the disease phenotype, may be sufficient to prevent or ameliorate symptoms of the disease. In some cases suppression of more than one component may be required to improve disease symptoms. This invention may

be applied in possible future interventive therapies for common polygenic diseases to suppress a particular genotype(s) using suppression and, when necessary, replacement steps.

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_____The present invention is exemplified using four genes: human rhodopsin, mouse rhodopsin, human peripherin and human collagen 1A2. The first of these genes are retinal specific. In contrast, collagen 1A2 is expressed in a range of tissues including skin and bone. While these four genes have been used as examples there is no reason why the invention could not be deployed in the suppression of many other genes in which mutations cause or predispose to a deleterious effect. Many examples of mutant genes ~~which~~ that give rise to disease phenotypes are available from the prior art -- these genes all represent targets for the invention. The present invention is exemplified using hammerhead ribozymes with antisense arms to elicit RNA cleavage. There is no reason why other suppression effectors

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directed towards genes, gene transcripts or gene products could not be used to achieve gene suppression. Many examples from the prior art detailing use of

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suppression effectors such as, inter alia, antisense RNA/DNA, triple helix forming DNA, PNAs and peptides to achieve suppression of gene expression are reported--(see

~~prior art~~). The present invention is exemplified using hammerhead ribozymes with antisense arms to elicit sequence specific cleavage of transcripts from genes implicated in dominant disorders and non--cleavage of transcripts from replacement genes containing sequence modifications in wobble positions such that the replacement gene still codes for wild type protein. The present invention is exemplified using suppression effectors targeting sites in coding regions of the human and mouse rhodopsin, human peripherin and human collagen 1A2 genes. Target sites, which include sequences from transcribed but untranslated regions of genes, introns, regions involved in the control of gene expression, regions adjacent to the gene or any combination of these, could be used to achieve gene suppression. Multiple suppression effectors, for example, shotgun ribozymes could be used to optimise efficiency of suppression when necessary. Additionally, when required, expression of a modified replacement gene such that the replacement gene product is altered from the wild type product such that it provides a beneficial effect may be used to provide gene product.

MATERIALS and METHODS

5

Cloning vectors

_____ cDNA templates and ribozymes were cloned into commercial expression vectors (pCDNA3, pZeoSV or pBluescript) ~~which~~ that enable expression in a test tube from T7, T3 or SP6 promoters or expression in mammalian cells from CMV or SV40 promoters. DNA inserts were cloned into the multiple cloning site (MCS) of these vectors typically at or near the terminal ends of the MCS to delete most of the MCS and thereby prevent any possible problems with efficiency of expression subsequent to cloning.

15

Sequencing protocols

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____ Clones containing template cDNAs and ribozymes were sequenced by ABI automated sequencing machinery using standard protocols.

5 Expression of RNAs

____ RNA was obtained from clones by *in vitro* transcription using a commercially available Ribomax expression system (Promega) and standard protocols. RNA
10 purifications were undertaken using the Bio--101 RNA purification kit or a solution of 0.3M sodium acetate and 0.2% SDS after isolation from polyacrylamide gels. Cleavage reactions were performed using standard protocols with varying $MgCl_2$ concentrations (0--15mM) at 37°C,
15 typically for 3 hours. Time points were performed at the predetermined optimal $MgCl_2$ concentrations for up to 5 hours. Radioactively labelled RNA products were obtained by incorporating α -- P^{32} rUTP (Amersham) in the expression
20 reactions (Gaughan et al. 1995). Labelled RNA products were run on polyacrylamide gels before cleavage reactions were undertaken for the purpose of RNA purification and subsequent to cleavage reactions to establish if RNA cleavage had been achieved. Cleavage reactions were undertaken with 5mM Tris--HCl pH8.0 and varying
25 concentrations of $MgCl_2$ at 37°C.

RNA secondary structures

_____ Predictions of the secondary structures of human and
5 mouse rhodopsin, human peripherin and human collagen 1A2
mRNAs were obtained using the RNAPlotFold program.
Ribozymes and antisense were designed to target areas of
the RNA that were predicted to be accessible to
suppression effectors, for example open loop structures.
10 The integrity of open loop structures was evaluated from
the 10 most probable RNA structures. Additionally,
predicted RNA structures for truncated RNA products were
generated and the integrity of open loops between full
length and truncated RNAs compared.

15 TEMPLATES and RIBOZYMES

Human Rhodopsin

Template cDNA

20 _____ The human rhodopsin cDNA (SEQ ID NO:1) was cloned
into the HindIII and EcoRI sites of the MCS of pCDNA3 in a
5' to -3' orientation allowing subsequent expression of
RNA from the T7 or CMV promoter in the vector. The full

length 5'UTR sequence was inserted into this clone using primer driven PCR mutagenesis and a HindIII (in pCDNA3) to BstEII (in the coding sequence of the human rhodopsin cDNA) DNA fragment.

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cDNA with altered sequence at a wobble position

_____The human rhodopsin hybrid cDNA with a single base alteration (SEQ ID NO:2), a C--->G change (at nucleotide 271 of SEQ ID NO:2) was introduced into human rhodopsin cDNA, using a HindIII to BstEII PCR cassette, by primer directed PCR mutagenesis. This sequence change occurs at a silent position -- it does not give rise to an amino acid substitution -- however it eliminates the ribozyme cleavage site (GUX ---->GUG). The hybrid rhodopsin was cloned into pCDNA3 in a 5' to 3' orientation allowing subsequent expression of RNA from the T7 or CMV promoter in the vector.

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Rhodopsin cDNA carrying a Pro23Leu adRP mutation

20 _____A human rhodopsin adRP mutation, a C---->T change (at codon 23; nucleotide 217 of SEQ ID NO:3) was introduced into human rhodopsin cDNA, using a HindIII to BstEII PCR cassette by primer directed PCR mutagenesis. This sequence change results in the substitution of a Proline for a Leucine residue. Additionally the nucleotide change

25

creates a ribozyme cleavage site (CCC--->CTC) (nucleotide 216--218 of SEQ ID NO:3). The mutated rhodopsin nucleic acid sequence was cloned into the HindIII and EcoRI sites of pCDNA3 in a 5' to 3' orientation allowing subsequent expression of RNA from the T7 or CMV promoter in the vector (SEQ ID NO:3).

Ribozyme constructs

10 A hammerhead ribozyme (termed Rz10 (SEQ ID NO:29) ~~(SEQ ID NO:4)~~) designed to target a large conserved open loop structure in the RNA from the coding regions of the gene was cloned subsequent to synthesis and annealing into the HindIII and XbaI sites of pCDNA3 again allowing expression of RNA from the T7 or CMV promoter in the
15 vector (SEQ ID NO:4). The target site was GUC (the GUX rule) at position 475--477 (nucleotides 369--371 of SEQ ID NO:1) of the human rhodopsin sequence. Note there is a one base mismatch in one antisense arm of Rz10. A
20 hammerhead ribozyme (termed Rz20 (SEQ ID NO:30) ~~)~~ designed to target an open loop structure in RNA from the coding region of a mutant rhodopsin gene with a Pro23Leu mutation was cloned subsequent to synthesis and annealing into the HindIII and XbaI sites of pCDNA3 again allowing expression of RNA from the T7 or CMV promoter in the vector (SEQ ID
25 NO:5). The target site was CTC (the NUX rule) at codon 23 (nucleotides 216--218 of SEQ ID NO:3) of the human

rhodopsin sequence (Accession number: K02281). Antisense
flanks are underlined.

Rz10: GGTCGGTCTGATGAGTCCGTGAGGACGAAACGTAGAG (SEQ ID NO:29;
nucleotides 101--137 of SEQ ID NO:4)

5 Rz20: TACTCGAACTGATGAGTCCGTGAGGACGAAAGGCTGC (SEQ ID NO:30;
nucleotides 104--140 of SEQ ID NO:5)

Mouse rhodopsin

10 Template cDNA

_____The full length mouse rhodopsin cDNA was cloned into
the EcoRI sites of the MCS of pCDNA3 in a 5' to 3'
orientation allowing subsequent expression of RNA from
the T7 or CMV promoter in the vector (SEQ ID NO:6).

15

cDNA with altered sequence at a wobble position

_____The mouse rhodopsin hybrid cDNA with a single base
alteration, a T--->C change (at position 1460)
(nucleotide 190 of SEQ ID NO:7) was introduced into mouse
20 rhodopsin cDNA, using a HindIII to Eco47III PCR cassette,
by primer directed PCR mutagenesis. This sequence change
occurs at a silent position -- it does not give rise to an

amino acid substitution -- however it eliminates the
ribozyme cleavage site (TTT--->TCT) (nucleotides 189--191
of SEQ ID NO:7). The hybrid rhodopsin was cloned into
pCDNA3 in a 5' to 3' orientation allowing subsequent
5 expression of RNA from the T7 or CMV promoter in the
vector (SEQ ID NO:7).

Ribozyme constructs

10 A hammerhead ribozyme (termed Rz33) (SEQ ID NO:31)
designed to target a large robust open loop structure in
the RNA from the coding regions of the gene was cloned
subsequent
to synthesis and annealing into the HindIII and XbaI sites
of pCDNA3 again allowing expression of RNA from the T7 or
15 CMV promoter in the vector (SEQ ID NO:8). The target site
was TTT (the NUX rule) at position 1459--1461 (nucleotides
405--407 of SEQ ID NO:6) of the mouse rhodopsin sequence.
(Accession number: M55171). Antisense flanks are
underlined.

20 Rz33: GGCACATCTGATGAGTCCGTGAGGACGAAAAAATTGG (SEQ ID NO:31;
nucleotides 118--154 of SEQ ID NO:8)

Human peripherin

Template cDNA

5 ___ The full length human peripherin cDNA was cloned into the EcoRI sites of the MCS of pCDNA3 in a 5' to 3' orientation allowing subsequent expression of RNA from the T7 or CMV promoter in the vector (SEQ ID NO:9).

10 DNAs with altered sequence at a wobble position

 ___ A human peripherin hybrid DNA with a single base alteration, a A--->G change (at position 257) (nucleotide 332 of SEQ ID NO:10) was introduced into human peripherin DNA by primer directed PCR mutagenesis (forward 257
15 mutation primer -- 5'CATGGCGCTGCTGAAAGTCA3' (SEQ ID NO:11) -- the reverse 257 primer was complementary to the forward primer). This sequence change occurs at a silent position -- it does not give rise to an amino acid substitution -- however it eliminates the ribozyme cleavage site (CTA---
20 >CTG) (nucleotide 330-332 of SEQ ID NO:10). A second human peripherin hybrid DNA with a single base alteration, a A-->G change (at position 359) (nucleotide 468 of SEQ ID NO:13) was introduced into human peripherin DNA, again by primer directed PCR mutagenesis (forward 359 mutation

primer -- 5'CATCTTCAGCCTGGGACTGT3' (SEQ ID NO:12) -- the reverse 359 primer was complementary to the forward primer) (SEQ ID NO:12). Similarly this sequence change occurs at a silent position -- it does not give rise to an amino acid substitution -- however again it eliminates the ribozyme cleavage site (CTA--->CTG) (nucleotides 466-468 of SEQ ID NO:13). The ribozyme cleavage sites at 255--257 (nucleotides 330--332 of SEQ ID NO:10) and 357--359 (nucleotides 466--468 of SEQ ID NO:13) occur at different open loop structures as predicted by the RNAPlotFold program. Hybrid peripherin DNAs included the T7 promoter sequence allowing subsequent expression of RNA.

Ribozyme constructs

15 Hammerhead ribozymes (termed Rz30 and Rz31) (SEQ ID NOS: 32 and 33, respectively), designed to target robust open loop structures in the RNA from the coding regions of the gene, were cloned subsequent

to synthesis and annealing into the HindIII and XbaI sites of pCDNA3 again allowing expression of RNA from the T7 or CMV promoter in the vector (SEQ ID NOS:14 and 15, respectively). The target sites were both CTA (the NUX rule) at positions 255--257 and 357--359 respectively of the human peripherin sequence. (Accession number: 25 M73531). Antisense flanks are underlined.

Rz30: ACTTTCAGCTGATGAGTCCGTGAGGACGAAAGCGCCA (SEQ ID NO:32;
nucleotides 116--153 of SEQ ID NO:14)

Rz31: ACAGTCCCTGATGAGTCCGTGAGGACGAAAGGCTGAA (SEQ ID NO:33;
nucleotides 112--148 of SEQ ID NO:15)

5

Human Type I Collagen -- COL1A2

Template cDNA

10 A human type I collagen 1A2 cDNA was obtained from
the ATCC (Accession No: Y00724). A naturally occurring
polymorphism has previously been found in collagen 1A2 at
positions 907 of the gene involving a T--->A nucleotide
change (Filie et al. 1993). The polymorphism occurs in a
predicted open loop structure of human collagen 1A2 RNA.
15 Polymorphic variants of human collagen 1A2 were generated
by PCR directed mutagenesis, using a HindIII to XbaI PCR
cassette. Resulting clones contained the following
polymorphism : collagen 1A2 (A) has a T nucleotide at
position 907 (A nucleotide 176 of SEQ ID NO:17, reverse
20 strand). In contrast human collagen 1A2 (B) has an A
nucleotide at position 907 (T nucleotide 181 of SEQ ID
NO:16, reverse strand). In collagen 1A2 (A) there is a
ribozyme target site, that is a GTC site (906--908)
(nucleotides 175-177 of SEQ ID NO:17, reverse strand),
25 however this cleavage site is lost in collagen 1A2 (B) as

the sequence is altered to GAC (906--908) (nucleotides 180--182 of SEQ ID NO:16, reverse strand), thereby disrupting the ribozyme target site.

5 Ribozyme constructs

_____ A hammerhead ribozyme (termed Rz907) (SEQ ID NO:34) was designed to target a predicted open loop structure in the RNA from the coding region of the polymorphic variant of the human collagen 1A2 gene. Rz907 oligonucleotide
10 primers were synthesised, annealed and cloned into the HindIII and XbaI sites of pCDNA3 again allowing subsequent expression of RNA from the T7 or CMV promoter in the vector (SEQ ID NO:18). The target site was a GUX site at
15 position 906--908 of the human type I collagen 1A2 sequence (Accession number: Y00724). _Antisense flanks are underlined.

Rz907: CGGCGGCTGATGAGTCCGTGAGGACGAAACCAGCA (SEQ ID NO:34; nucleotide 107--141 of SEQ ID NO:18)

FIGURE LEGENDS

~~Diagram~~ Figure 1:

5 pBR322 was cut with MspI, radioactively
labeledlabelled and run on a polyacrylamide gel to enable
separation of the resulting DNA fragments. The sizes of
these fragments are given in ~~diagram~~ Figure 1. This DNA
ladder was then used on subsequent polyacrylamide gels
10 (4--8%) to provide an estimate of the size of the RNA
products run on the gels. However there is a significant
difference in mobility between DNA and RNA depending on
the percentage of polyacrylamide and the gel running
conditions -- hence the marker provides an estimate of
15 size of transcripts.

Figure 2:1

A: Human rhodopsin cDNA (SEQ ID NO:1) was expressed from
the T7 promoter to the BstEII site in the coding sequence.
20 Resulting RNA was mixed with Rz10RNA in 15mM magnesium
chloride and incubated at 37°C for varying times. Lanes
1--4: Human rhodopsin RNA and Rz10RNA after incubation at
37°C with 15mM magnesium chloride for 0, 1 2 and 3 hours
respectively. Sizes of the expressed RNAs and cleavage

products are as expected (Table 1). Complete cleavage of human rhodopsin RNA was obtained with a small residual amount of intact RNA present at 1 hour. Lane 6 is intact unadapted human rhodopsin RNA (BstEII) alone. Lane 5 is unadapted human rhodopsin RNA (FspI) alone and refers to Figure 1B2B. From top to bottom, human rhodopsin RNA and the two cleavage products from this RNA are highlighted with arrows.

B: The unadapted human rhodopsin cDNA was expressed from the T7 promoter to the FspI site in the coding sequence. The adapted human rhodopsin cDNA was expressed from the T7 promoter to the BstEII site in the coding sequence. Lanes 1--4: Resulting RNAs were mixed together with Rz10 and 15mM magnesium chloride and incubated at 37°C for varying times (0, 1, 2 and 3 hours respectively). The smaller unadapted rhodopsin transcripts were cleaved by Rz10 while the larger adapted transcripts were protected from cleavage by Rz10. Cleavage of adapted protected transcripts would have resulted in products of 564bases and 287bases -- the 564bases product clearly is not present -- the 287bp product is also generated by cleavage of the unadapted human rhodopsin ~~transcripts~~transcripts and hence is present (FspI). After 3 hours the majority of the unadapted rhodopsin transcripts has been cleaved by Rz10. Lane 5 contains the intact adapted human rhodopsin RNA (BstEII) alone. From top to bottom adapted uncleaved human rhodopsin transcripts, residual unadapted uncleaved human rhodopsin transcripts and the larger of the cleavage

products from unadapted human rhodopsin transcripts are highlighted by arrows. The smaller 22 bases cleavage product from the unadapted human rhodopsin transcripts has run off the gel.

5 | Figure 3:-2

A: Unadapted (SEQ ID NO:1) and adapted (SEQ ID NO:2) human rhodopsin cDNAs were expressed from the T7 promoter to the AcyI after the coding sequence and the BstEII site in the coding sequence respectively. Sizes of expressed
10 RNAs and cleavage products were as predicted (Table 1). Resulting RNAs were mixed together with Rz10 RNA at varying magnesium chloride concentrations and incubated at 37°C for 3 hours. Lane 1: Intact unadapted human
15 | rhodopsin RNA (AcyI) alone. Lanes 2--5: Unadapted and adapted human rhodopsin RNAs and Rz10 RNA after incubation at 37°C with 0, 5, 10 and 15 mM MgCl₂ respectively. Almost complete cleavage of the larger unadapted human rhodopsin RNA was obtained with a small residual amount of intact RNA present at 5 mM MgCl₂. In contrast the adapted
20 human rhodopsin RNA remained intact. From top to bottom, the unadapted and adapted rhodopsin RNAs, and two cleavage products from the unadapted human rhodopsin RNA are highlighted by
| arrows. Lane 6 is intact adapted human rhodopsin RNA
25 (BstEII) alone.

B: The adapted human rhodopsin cDNA was expressed from the T7 promoter to the BstEII site in the coding sequence.

Lanes 1--4: Resulting RNA was mixed together with Rz10 and 0, 5, 10 and 15 mM magnesium chloride and incubated at 37°C for 3 hours respectively. The adapted rhodopsin transcripts were not cleaved by Rz10. Cleavage of adapted transcripts would have resulted in cleavage products of 564 bases and 287 bases which clearly are not present. Lane 5: intact adapted human rhodopsin RNA (BstEII) alone. Lane 4: RNA is absent -- due to a loading error or degradation. The adapted uncleaved human rhodopsin RNA is highlighted by an arrow.

C: Unadapted (SEQ ID NO:1) and adapted (SEQ ID NO:2) human rhodopsin cDNAs were expressed from the T7 promoter to the AcyI after the coding sequence and the BstEII site in the coding sequence respectively. Sizes of expressed RNAs and cleavage products were as predicted (Table 1). Resulting RNAs were mixed together with Rz10 RNA at varying magnesium chloride concentrations and incubated at 37°C for 3 hours. Lane 1: DNA ladder as in Diagram Figure 1. Lanes 2--5: Unadapted and adapted human rhodopsin RNAs and Rz10 RNA after incubation at 37°C with 0, 5, 10 and 15 mM MgCl₂ respectively. Almost complete cleavage of the larger unadapted human rhodopsin RNA was obtained with a small residual amount of intact RNA present at 5 and 10 mM MgCl₂. In contrast the adapted human rhodopsin RNA remained intact. Lane 6: Adapted human rhodopsin RNA (BstEII) alone. Lane 7: Unadapted

human rhodopsin RNA (AcyI) alone. Lane 8: DNA ladder as in ~~Diagram~~ Figure 1. From top to bottom, the unadapted and adapted rhodopsin RNAs, and two cleavage products from the unadapted human rhodopsin RNA are highlighted by
5 arrows. Separation of the adapted human rhodopsin RNA (851 bases) and the larger of the cleavage products from the unadapted RNA (896 bases) is incomplete in this gel (further ~~running~~ running of the gel would be required to achieve separation) -- however the separation of these
10 two RNAs is demonstrated in Figure 32A.

Figure 4:3

 The mutant (Pro23Leu) (SEQ ID NO:3) human rhodopsin cDNA was expressed from the T7 promoter to the BstEII in
15 the coding sequence. Likewise the Rz20 clone was expressed to the XbaI site. Resulting RNAs were mixed together with 10mM magnesium chloride concentrations at 37°C for varying times. Sizes of expressed RNAs and cleavage products were as predicted (Table 1). Lane 1:
20 DNA ladder as in ~~Diagram~~ Figure 1. Lanes 2: Pro23Leu human rhodopsin RNA alone. Lanes 3--7 Pro23Leu human rhodopsin RNA and Rz20 RNA after incubation at 37°C with 10 mM MgCl₂ for 0mins, 30 mins, 1 hr, 2hrs and 5hrs respectively. Almost complete cleavage of mutant
25 rhodopsin transcripts was obtained with a residual amount of intact RNA left even after 5 hours. Lane

8: DNA ladder as in ~~Diagram~~ Figure 1. From top to bottom, intact mutant rhodopsin RNA and the two cleavage products from the mutant human rhodopsin RNA are highlighted by arrows.

5

Figure 5:4

The mutant (Pro23Leu) (SEQ ID NO:3) human rhodopsin cDNA was expressed from the T7 promoter to the BstEII in the coding sequence. Likewise the Rz10 clone (SEQ ID NO:4) was expressed to the XbaI site. Resulting RNAs were mixed together with 10mM magnesium chloride concentrations at 37°C for varying times. Sizes of expressed RNAs and cleavage products were as predicted (Table 1). Lane 1: DNA ladder as in ~~Diagram~~ Figure 1. Lanes 2: Pro23Leu human rhodopsin RNA alone. Lanes 3--7 Pro23Leu human rhodopsin RNA and Rz10 RNA after incubation at 37°C with 10 mM MgCl₂ for 0mins, 30 mins, 1 hr, 2hrs and 5hrs respectively. Almost complete cleavage of mutant human rhodopsin RNA was obtained with a residual amount of intact RNA remaining even after 5 hours (Lane 7). Lane 8: DNA ladder as in ~~Diagram~~ Figure 1. From top to bottom, intact mutant rhodopsin RNA and the two cleavage products from the mutant human rhodopsin RNA are highlighted by arrows.

Figure 6:5

_____The mouse rhodopsin cDNA clone was expressed *in vitro* from the T7 promoter to the Eco47III site in the coding sequence. Likewise the Rz33 clone was expressed to the XbaI site. A: Resulting RNAs were mixed together with 10mM magnesium chloride at 37°C for varying times. Sizes of expressed RNAs and cleavage products were as predicted (Table 1). DNA ladder as in ~~Diagram~~ Figure 1. Lane 1: mouse rhodopsin RNA alone. Lanes 2--5 Mouse rhodopsin RNA and Rz33 RNA after incubation at 37°C with 10 mM MgCl₂ at 0, 5, 7.5 and 10 mM MgCl₂ respectively for 3 hours. Cleavage of mouse rhodopsin RNA was obtained after addition of divalent ions (Lane 3). Residual uncleaved mouse rhodopsin RNA and the two cleavage products from the mouse rhodopsin RNA are highlighted by arrows. B: The adapted mouse rhodopsin cDNA clone with a base change at position 1460 (nucleotide 190 of SEQ ID NO:7) was expressed *in vitro* from the T7 promoter to the Eco47III site in the coding sequence. Likewise the Rz33 clone was expressed to the XbaI site. Resulting RNAs were mixed together with various magnesium chloride concentrations at 37°C for 3 hours. Sizes of expressed RNAs and cleavage products were as predicted (Table 1). Lane 1: DNA ladder as in ~~Diagram~~ Figure 1. Lane 2: Adapted mouse rhodopsin RNA alone. Lanes 3--6: Adapted mouse rhodopsin RNA and Rz33 RNA after incubation at 37°C with 0, 5, 7.5 and 10 mM

MgCl₂ for 3 hours at 37°C. No cleavage of the adapted mouse rhodopsin RNA was observed. The intact adapted mouse rhodopsin RNA is highlighted by an arrow.

5 Figure 7:6

_____ The human peripherin cDNA clone was expressed in vitro from the T7 promoter to the BglII site in the coding sequence. Likewise Rz30 (targeting a cleavage site at 255--257) was expressed to the XbaI site. A:

10 Resulting RNAs were mixed together with 10mM magnesium chloride at 37°C for varying times. Lane 1: DNA ladder as in ~~Diagram-Figure 1~~. Lane 2: Intact human peripherin RNA alone. Lanes 3--7: Human peripherin RNA and Rz30 RNA after incubation at 37°C with 10 mM MgCl₂ for 3hrs, 2hrs, 15 1hr, 30 mins and 0 mins respectively. Lane 8: DNA ladder as in ~~Diagram-Figure 1~~. From top to bottom, intact human peripherin RNA and the two cleavage products from the human peripherin RNA are highlighted by arrows. B:

20 Resulting RNAs were mixed with Rz30 RNA at varying magnesium chloride concentrations and incubated at 37°C for 3hrs. Lane 1: DNA ladder as in ~~Diagram-Figure 1~~. Lanes 2--5: Human peripherin RNA and Rz30 after incubation at 37°C with 10, 7.5, 5 and 0 mM magnesium chloride respectively for 3hrs. Lane 6: Intact human peripherin 25 RNA alone. Sizes of the expressed RNAs and cleavage

products are as expected (Table 1). Significant cleavage of human peripherin RNA was obtained with a residual amount of intact RNA present at each $MgCl_2$ concentration. From top to bottom, human peripherin RNA and the two cleavage products from this RNA are highlighted with arrows. C: The adapted human peripherin DNA with a single base change at position 257 was expressed from the T7 promoter to the AvrII site in the coding sequence. Resulting RNA was mixed with Rz30 at various magnesium chloride concentrations and incubated at 37°C for 3hrs.

10 Lane 1: DNA ladder as in ~~Diagram~~ Figure 1. Lane 2: Intact adapted human peripherin RNA alone. Lanes 3--6: Adapted human peripherin RNA and Rz30 after incubation at 37°C with 0, 5, 7.5 and 10 mM magnesium chloride respectively for 3hrs. Lane 7: DNA ladder as in ~~Diagram~~ Figure 1. D: The unadapted human peripherin cDNA and the adapted human peripherin DNA fragment with a single base change at position 257 were expressed from the T7 promoter to the BglII and AvrII sites respectively in the coding sequence.

15 Resulting RNAs were mixed with Rz30 at various magnesium chloride concentrations and incubated at 37°C for 3hrs.

20 Lane 1: DNA ladder as in ~~Diagram~~ Figure 1. Lane 2: Intact unadapted human peripherin RNA alone. Lane 3: Intact adapted human peripherin RNA alone. Lanes 4--7: Unadapted and adapted human peripherin RNAs and Rz30 after incubation at 37°C with 0, 5, 7.5 and 10 mM magnesium chloride respectively for 3hrs at 37°C. No cleavage of the adapted human peripherin RNA was observed even after 3

hours whereas a significant reduction in the unadapted RNA was observed over the same time frame. Lane 8: DNA ladder as in ~~Diagram~~-Figure 1. From top to bottom, residual unadapted human peripherin RNA, adapted human peripherin RNA and the two cleavage products are highlighted by arrows.

Figure 8:7

Human peripherin cDNA clone was expressed *in vitro* from the T7 promoter to the BglII site in the coding sequence. Likewise the Rz31 (targeting a cleavage site at 357--359) (nucleotides 466--468 of SEQ ID NO:13) was expressed to the XbaI site. A: Resulting RNAs were mixed together with 10mM magnesium chloride at 37°C for varying times. Lane 1: DNA ladder as in ~~Diagram~~-Figure 1. Lanes 2--6: Human peripherin RNA and Rz31 RNA after incubation at 37°C with 10mM MgCl₂ for 3hrs, 2hrs, 1hr, 30mins and 0mins respectively. Increased cleavage of mouse rhodopsin RNA was obtained over time -- however significant residual intact RNA remained even after 3 hours (Lane 2). Lane 7: Intact human peripherin RNA alone. Lane 8: DNA ladder as in ~~Diagram~~-Figure 1. From top to bottom, intact human peripherin RNA and the two cleavage products from the human peripherin RNA are highlighted by arrows. B: Resulting RNAs were mixed with Rz31 RNA at varying magnesium chloride concentrations and incubated at 37°C

for 3hrs. Lane 1: DNA ladder as in ~~Diagram~~ Figure 1.
Lanes 2--5: Human peripherin RNA and Rz31 after incubation
at 37°C with 10, 7.5, 5 and 0_m-M magnesium chloride
respectively for 3hrs. Sizes of the expressed RNAs and
5 cleavage products are as expected (Table 1). Significant
cleavage of human peripherin RNA was obtained with a
residual amount of intact RNA present at each MgCl₂
concentration (Lanes 2--4). Lane 6: Intact human
peripherin RNA alone. Lane 7: DNA ladder as in ~~Diagram~~
10 Figure 1. From top to bottom, human peripherin RNA and
the two cleavage products from this RNA are highlighted
with arrows. C: The adapted human peripherin DNA with a
single base change at position 359 (nucleotide 468 of SEQ
ID NO:13) was expressed from the T7 promoter to the BglII
15 site in the coding sequence. Resulting RNA was mixed with
Rz31 at various magnesium chloride concentrations and
incubated at 37°C for 3hrs. Lane 1: DNA ladder as in
~~Diagram~~ Figure 1. Lane 2: Intact adapted human peripherin
RNA alone. Lanes 3--6: Adapted human peripherin RNA and
20 Rz31 after incubation at 37°C with 0, 5, 7.5 and 10mM
magnesium chloride respectively for 3hrs. No cleavage of
the adapted human peripherin RNA was observed even after 3
hours. Lane 7: DNA ladder as in ~~Diagram~~ Figure 1. The
~~sequence quality was not good in the region of the human~~
25 ~~peripherin 359 silent change (nucleotide 468 of SEQ ID~~
~~NO:13) the sequencing primer was too far from the target~~
~~site to achieve good quality sequence (SEQ ID NO: 13).~~

Figure 9:8

A: The human collagen 1A2 cDNA clones containing the A and T alleles of the polymorphism at position 907 were expressed from the T7 promoter to the MvnI and XbaI sites in the insert and vector respectively. Resulting RNAs were mixed together with Rz907 and various MgCl₂ concentrations and incubated at 37°C for 3 hours. Lane 1: intact RNA from the human collagen 1A2 (A) containing the A allele of the 907 polymorphism. Lane 2: intact RNA from the human collagen 1A2 (B) containing the T allele of the 907 polymorphism. Lanes 3--5: Human collagen 1A2 (A) and (B) representing the A and T allele RNAs and Rz907 incubated with 0, 5, and 10 mM MgCl₂ at 37°C for 3 hours. RNA transcripts from the T allele containing the 906--908 target site are cleaved by Rz907 upon addition of divalent ions -- almost complete cleavage is obtained with a residual amount of transcript from the T allele remaining (Lane 5). In contrast transcripts expressed from the A allele (which are smaller in size to distinguish between the A (MvnI) and T (XbaI) alleles) were not cleaved by Rz907 -- no cleavage products were observed. From top to bottom, RNA from the T allele, the A allele and the two cleavage products from the T allele are highlighted by arrows. Lane 6: DNA ladder as in ~~Diagram~~ Figure 1.

B: The human collagen 1A2 cDNA (A) + (B) clones containing the A and T alleles of the polymorphism at 907 were expressed from the T7 promoter to the MvnI and XbaI sites in the insert and vector respectively. Resulting RNAs were mixed together with Rz907 and 10mM magnesium chloride and incubated at 37°C for varying times. Lane 1: DNA ladder as in ~~Diagram~~ Figure 1. Lane 2: intact RNA from the human collagen 1A2 (A) with the A allele of the 907 polymorphism. Lane 3: intact RNA from the human collagen 1A2 (B) with the T allele of the 907 polymorphism. Lanes 4--9: Human collagen 1A2 A and T allele RNA and Rz907 incubated with 10mM MgCl₂ at 37°C for 0, 30 mins, 1 hour, 2 hours, 3 hours and 5 hours respectively. RNA transcripts from the T allele containing the 906--908 target site are cleaved by Rz907 -- almost complete cleavage is obtained after 5 hours. In contrast transcripts expressed from the A allele (which are smaller in size to distinguish between the A (MvnI) and T (XbaI) alleles) were not cleaved by Rz907 -- no cleavage products were observed. From top to bottom, RNA from the T allele, the A allele and the two cleavage products from the T allele are highlighted by arrows.

Figure 10:

The human rhodopsin cDNA in pcDNA3. (SEQ ID NO: 1).

Figure 11:

The human rhodopsin cDNA in pcDNA3 (SEQ ID NO:2) with a base change at a silent site (477) (nucleotide 271 of SEQ ID NO:2).

5 Figure 12:

Mutant (Pro23Leu) (nucleotides 216--218 of SEQ ID NO:3) human rhodopsin cDNA in pcDNA3 (SEQ ID NO:3).

Figure 13:

10 Rz10 cloned into pcDNA3 (SEQ ID NO:4). . Note there is a one base mismatch in one antisense arm of Rz10.

Figure 14:

Rz20 cloned into pcDNA3 (SEQ ID NO:5).

Figure 15:

The mouse rhodopsin cDNA in pcDNA3 (SEQ ID NO:6).

15 Figure 16:

The mouse rhodopsin cDNA in pcDNA3 (SEQ ID NO:7) with a base change at a silent site (1460) (nucleotide 190 of SEQ ID NO:7).

Figure 17:

20 Rz33 cloned into pcDNA3 (SEQ ID NO:8)

Figure 18:

The human peripherin cDNA in pCDNA3 (SEQ ID NO:9).

Figure 19:

5 The human peripherin DNA fragment (SEQ ID NO:10) with a
base change at a silent site (257) (nucleotide 332 of SEQ
ID NO:10).

Figure 20:

10 The human peripherin DNA fragment (SEQ ID NO:11) with a
base change at a silent site (359) (nucleotide 468 of SEQ
ID NO:13). The sequence quality was not good in the region
of the human peripherin 359 silent change (nucleotide 468
of SEQ ID NO:13) - the sequencing primer was too far from
the target site to achieve good quality sequence.

Figure 21:

15 Rz30 cloned into pCDNA3 (SEQ ID NO:12)

Figure 22:

Rz31 cloned into pCDNA3 (SEQ ID NO:13)

Figure 23:

20 Collagen 1A2 (A) sequence containing the A polymorphism at
position 907. (SEQ ID NO:14) (Note there is an
additional polymorphic site at position 902).

Figure 24:

Collagen 1A2 (B) sequence containing the T polymorphism at position 907. (SEQ ID NO:15) (Note there is an additional polymorphic site at position 902).

5 Figure 25:

Rz907 cloned into pcDNA3 (SEQ ID NO:18)

RESULTS

10 _____ Human and mouse rhodopsin, human peripherin and human collagen 1A2 cDNA clones were expressed *in vitro*.

Ribozymes targeting specific sites in the human and mouse rhodopsin, human ~~peipherin~~peripherin and human collagen 1A2 cDNAs were also expressed *in vitro*. cDNA clones were cut with various restriction enzymes resulting in the production of differently sized transcripts after expression. This aided in differentiating between RNAs expressed from unadapted and adapted cDNAs. Restriction enzymes used to cut each clone, sizes of resulting transcripts and predicted sizes of products after cleavage by target ribozymes are given below in Table 1. Exact sizes of expression products may vary by a few bases from that estimated as there may be some ambiguity concerning

15

20

inter alia the specific base at which transcription starts.

Example 1:

5

A: Human Rhodopsin

_____The unadapted human rhodopsin cDNA (SEQ ID NO:1) and the human rhodopsin cDNA with a single nucleotide substitution in the coding sequence (SEQ ID NO:2) were cut with BstEII and expressed *in vitro*. The single base change occurs at the third base position or wobble position of the codon (at position 477) (nucleotide 271 of SEQ ID NO:2) and therefore does not alter the amino acid coded by this triplet. The Rz10 clone was cut with XbaI and expressed *in vitro*. Resulting ribozyme and human rhodopsin RNAs were mixed with varying concentrations of MgCl₂ to optimise cleavage of template RNA by Rz10. A profile of human rhodopsin RNA cleavage by Rz10 over time is given in Figure 2A. The MgCl₂ curve profile used to test if adapted human rhodopsin transcripts could be cleaved by Rz10 is given in Figure 3B. Unadapted and adapted human rhodopsin cDNAs were cut with FspI and BstEII respectively, expressed and mixed together with Rz10 RNA to test for cleavage (Figure 2B) over time.

Likewise, unadapted and adapted human rhodopsin cDNAs were cut with AcyI and BstEII respectively, both were expressed *in vitro* and resulting transcripts mixed with Rz10 RNA at varying $MgCl_2$ concentrations to test for cleavage (Figure 5 | 32A, 23C). In all cases expressed RNAs were the predicted size. Similarly in all cases unadapted transcripts were cleaved into products of the predicted size. Cleavage of unadapted human rhodopsin RNA was almost complete -- little residual uncleaved RNA remained. In all cases 10 adapted human rhodopsin RNAs with a single base change at a silent site remained intact, that is, they were not cleaved by Rz10. Clearly, transcripts from the unadapted human rhodopsin cDNA are cleaved by Rz10 while transcripts from the adapted replacement gene which has been modified 15 in a manner which exploits the degeneracy of the genetic code are protected from cleavage. It is worth noting that AcyI enzyme cuts after the stop codon and therefore the resulting RNA includes the complete coding sequence of the gene.

20 |

B: Human Rhodopsin

| _____ Rz20 was cut with XbaI and expressed *in vitro*.
Similarly the rhodopsin cDNA containing a Pro23Leu
25 | mutation was cut with BstEII and expressed *in vitro*.

Resulting RNAs were mixed and incubated at 37°C with 10mM MgCl₂ for varying times. Rz20 was designed to elicit mutation specific cleavage of transcripts containing a Pro23Leu rhodopsin mutation. All expressed products and
5 | cleavage products were the correct size. Figure 3-4 demonstrates mutation specific cleavage of the mutant RNA over time incubated at 37°C with 10mM MgCl₂. Cleavage of mutant rhodopsin transcripts by Rz10 which targets a ribozyme cleavage site 3' of the site of the Pro23Leu
10 | mutation in rhodopsin coding sequence was explored. The mutant rhodopsin cDNA and Rz10 clones were cut with BstEII and XbaI respectively and expressed *in vitro*. Resulting RNAs were mixed and incubated with 10mM MgCl₂ for varying
15 | times (Figure 54). All expressed products and cleavage products were the correct size. Rz10 cleaved mutant rhodopsin transcripts. Using a replacement gene with a sequence change around the Rz10 cleavage site which is at a wobble position we demonstrated in Example 1A that transcripts from the replacement gene remain intact due to
20 | absence of the Rz10 target site (Figures 2-1B, 3-2A and 3-2B). Hence Rz10 could be used to cleave mutant transcripts in a manner independent of the disease mutation itself (that is, using this site) while transcripts from the replacement gene which code for the
25 | correct protein would remain intact and therefore could supply the wild type protein.

Example 2:

Mouse Rhodopsin

_____Rz33 was cut with XbaI and expressed *in vitro*.

- 5 Similarly the mouse rhodopsin cDNA was cut with Eco47III and expressed *in vitro*. Resulting RNAs were mixed and incubated with varying concentrations of $MgCl_2$. All expressed products and cleavage products were the correct size. Figure 65A demonstrates specific cleavage of the
- 10 mouse rhodopsin RNA over various $MgCl_2$ concentrations incubated at 37°C for 3 hours. Using a replacement gene with a sequence change around the Rz33 cleavage site (TTT--->TCT) (nucleotides 189--191 of SEQ ID NO:7) which is at a wobble position we demonstrated that transcripts
- 15 from the replacement gene remain intact due to absence of the Rz33 target site (Figures 65B). Hence Rz33 could be used to cleave mutant transcripts in a manner independent of the
- 20 disease mutation itself (that is, using this site) while transcripts from the replacement gene which code for the correct protein would remain intact and therefore could supply the wild type protein.

Example 3:

Human Peripherin

_____The unadapted human peripherin cDNA and two human
5 peripherin DNA fragments generated by PCR mutagenesis with
a single nucleotide substitution in the coding sequence
were cut with BglII and AvrII respectively and expressed
in vitro. The single base changes in the adapted DNAs
occur at third base positions or wobble positions of the
10 codon (at position 257 and 359) (nucleotide 468 of SEQ ID
NO:13 and nucleotide 332 of SEQ ID NO:10, respectively)
and therefore do not alter the amino acid coded by these
triplets. The Rz30 and Rz31 clones were cut with XbaI and
expressed *in vitro*. Resulting ribozymes and unadapted
15 human rhodopsin RNAs were mixed with varying
concentrations of $MgCl_2$ to optimise cleavage of template
RNA by Rz30 and Rz31. Profiles of human
~~perpherin~~peripherin RNA cleavage by Rz30 over various
 $MgCl_2$ concentrations and over time are given in Figure 76.
20 Similarly profiles of human ~~perpherin~~peripherin RNA
cleavage by Rz31 over various $MgCl_2$ concentrations and
over time are given in Figure 87. In all cases expressed
RNAs were the predicted size. Similarly in all cases
unadapted transcripts were cleaved into products of the
25 predicted size. Adapted human rhodopsin RNAs were mixed
together with Rz30 and Rz31 RNA over various $MgCl_2$
concentrations to test if adapted human peripherin

transcripts could be cleaved by Rz30 and Rz31 (Figures 7 and 86+7). Expressed RNAs were the predicted size. In all cases adapted human peripherin RNAs with single base changes at silent sites remained intact, that is, they were not cleaved by Rz30 or Rz31. Clearly, transcripts from the unadapted human peripherin cDNA are cleaved by Rz30 and Rz31 while transcripts from the adapted replacement DNAs which have been modified in a manner which exploits the degeneracy of the genetic code are protected from cleavage.

Example 4:

__Human Collagen 1A2

____Rz907 clones targeting a polymorphic site in human collagen 1A2 sequence was cut with XbaI and expressed in vitro. The human collagen 1A2 cDNA clones (A and B)

__containing two allelic forms of a polymorphism in the coding sequence of the gene at positions 907 were cut with MvnI and XbaI respectively, expressed in vitro and RNAs mixed together with Rz907 RNA to test for cleavage of transcripts by this ribozyme. All expressed transcripts were of the predicted sizes. RNAs were mixed with varying concentrations of MgCl₂ to optimise cleavage of RNAs by Rz907 (Figure 98). Notably the majority of the RNA transcripts from human collagen 1A2 (A) which has a T nucleotide at position 907 (A nucleotide 176 of SEQ ID

NO:17, reverse strand) is cleaved by Rz907 (Figure 98). This allelic form of the gene has a ribozyme cleavage site at 906--908. Notably the situation is reversed with transcripts from human collagen 1A2 (B) where in this allelic form of the gene due to the nature of the polymorphism present at position 907 the ribozyme cleavage site has been lost.

____ In contrast to transcripts from human collagen (A), transcripts from human collagen (B) were protected from cleavage by Rz907 due to the alteration in the sequence around the ribozyme cleavage site (Figure 98). Cleavage of collagen 1A2 (A) by Rz907 was efficient which is consistent with 2--D predictions of RNA open loop structures for the polymorphism -- in the allele containing the Rz907 ribozyme cleavage site, the target site is found quite consistently in an open loop structure. This polymorphism found in an open loop structure of the transcript clearly demonstrates the feasibility and utility of using the degeneracy of the genetic code in the suppression of an endogenous gene (either suppressing both alleles or a single allele at a polymorphic site) and restoration of gene expression using a gene which codes for the same protein but has sequence modifications at third base wobble positions which protect the replacement gene from suppression.

TABLE 1

		Restriction Enzyme	RNA Size	Cleavage Products
5	<u>Example 1</u>			
	Human rhodopsin	BstEII	851 bases	287+564 bases
		AcyI	1183 bases	287+896 bases
10		FspI	309 bases	287+22
	Adapted Human rhodopsin	BstEII	851 bases	
	Human rhodopsin			
	Pro--Leu	BstEII	851 bases	170+681 (Rz20)
15	Human rhodopsin			
	Pro--Leu	BstEII	851 bases	287+564 (Rz10)
	Rz10	XbaI	52 bases	
	Rz20	XbaI	52 bases	
	(Table 1; SEQ ID NOS:1--5 ; Figures 1--54)			
20	<u>Example 2</u>			
	Mouse rhodopsin	Eco47III	774 bases	400+374
	Adapted mouse rhodopsin	Eco47III	774 bases	
25		XbaI	52 bases	
	Rz33			
	(Table 1; SEQ ID NOS:6--9; Figure 65)			
30	<u>Example 3</u>			
	Human peripherin	BglII	545 bases	315+230 (Rz30)
	Human peripherin	BglII	545 bases	417+128 (Rz31)
	Adapted human peripherin	AvrII	414 bases	
35	Adapted human peripherin	BglII	545 bases	
	Rz30	XbaI	52 bases	
	Rz31	XbaI	52 bases	
	(Table 1; SEQ ID NOS:10, 13--16; Figures 6+77 and 8)			
40	<u>Example 4</u>			

	Human Collagen 1A2		
	(B) --Rz907	XbaI	888 bases 690+198 bases
	Human Collagen		
	1A2 (A)	MvnI	837 bases
5	Rz907	XbaI	52 bases
	(Table 1; SEQ ID NOS:16--18; Figure 89)		

TABLE 2

5	A: Rhodopsin mutations tested to assess if the —predicted open loop RNA structure containing the Rz10 target site (475--477) remains intact in mutant transcripts.														
10	<table> <tr> <th>Rhodopsin mutation</th><th>RNA open loop targeted by Rz10</th></tr> <tr><td>Pro 23 Leu</td><td>Intact</td></tr> <tr><td>Gly 51 Val</td><td>Intact</td></tr> <tr><td>Thr 94 Ile</td><td>Intact</td></tr> <tr><td>Gly 188 Arg</td><td>Intact</td></tr> <tr><td>Met 207 Arg</td><td>Intact</td></tr> <tr><td>Ile del 255</td><td>Intact</td></tr> </table>	Rhodopsin mutation	RNA open loop targeted by Rz10	Pro 23 Leu	Intact	Gly 51 Val	Intact	Thr 94 Ile	Intact	Gly 188 Arg	Intact	Met 207 Arg	Intact	Ile del 255	Intact
Rhodopsin mutation	RNA open loop targeted by Rz10														
Pro 23 Leu	Intact														
Gly 51 Val	Intact														
Thr 94 Ile	Intact														
Gly 188 Arg	Intact														
Met 207 Arg	Intact														
Ile del 255	Intact														
15	B: Utilisation of the degeneracy of the genetic code. Ribozyme cleavage sites are underlined														
20	<table> <tr><td>Human rhodopsin</td><td></td></tr> <tr><td>Unadapted sequence</td><td> <div> <div>—475--477</div> <div>TAC <u>GTC</u> ACC GTC CAG (SEQ ID NO:19)</div> <div>—Val</div> </div> </td></tr> </table>	Human rhodopsin		Unadapted sequence	<div> <div>—475--477</div> <div>TAC <u>GTC</u> ACC GTC CAG (SEQ ID NO:19)</div> <div>—Val</div> </div>										
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30	<table> <tr><td>Mouse rhodopsin</td><td></td></tr> <tr><td>Unadapted sequence</td><td> <div> <div>1459--1461</div> <div>AAT <u>TTT</u> TAT GTG CCC (SEQ ID NO:21)</div> <div>—Phe</div> </div> </td></tr> </table>	Mouse rhodopsin		Unadapted sequence	<div> <div>1459--1461</div> <div>AAT <u>TTT</u> TAT GTG CCC (SEQ ID NO:21)</div> <div>—Phe</div> </div>										
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35	<table> <tr><td>Adapted sequence</td><td> <div> <div>1459--1461</div> <div>AAT TTC TAT GTG CCC (SEQ ID NO:22)</div> <div>—Phe</div> </div> </td></tr> </table>	Adapted sequence	<div> <div>1459--1461</div> <div>AAT TTC TAT GTG CCC (SEQ ID NO:22)</div> <div>—Phe</div> </div>												
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45	<table> <tr><td>Unadapted sequence</td><td> <div> <div>—357--359</div> <div>AGC <u>CTA</u> GGA CTG TTC (SEQ ID NO:25)</div> <div>—Leu</div> </div> </td></tr> <tr><td>Adapted sequence</td><td> <div> <div>—357--359</div> <div>AGC CTG GGA CTG TTC (SEQ ID NO:26)</div> <div>—Leu</div> </div> </td></tr> </table>	Unadapted sequence	<div> <div>—357--359</div> <div>AGC <u>CTA</u> GGA CTG TTC (SEQ ID NO:25)</div> <div>—Leu</div> </div>	Adapted sequence	<div> <div>—357--359</div> <div>AGC CTG GGA CTG TTC (SEQ ID NO:26)</div> <div>—Leu</div> </div>										
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50	<table> <tr><td>Human type I collagen 1A2</td><td> <div> <div>—906--908</div> </div> </td></tr> </table>	Human type I collagen 1A2	<div> <div>—906--908</div> </div>												
Human type I collagen 1A2	<div> <div>—906--908</div> </div>														

Sequence (A)	GCT GGT CCC GCC GGT (SEQ ID NO:27)
	—Gly
	—906—908
Sequence (B)	GCT GGA CCC GCC GGT (SEQ ID NO:28)
	—Gly

DISCUSSION

In the examples outlined above, RNA was expressed from cDNAs coding for four different proteins: human and mouse rhodopsin, human peripherin and human type I collagen 1A2. Rhodopsin and peripherin have been used to exemplify the invention for retinopathies such as adRP -- suppression effectors have been targeted to the coding sequences of these genes. In the case of the human collagen 1A2 gene a naturally occurring polymorphism has been used to demonstrate the invention and the potential use of the invention for disorders such as OI -- however non--polymorphic regions of the collagen 1A2 gene could be used to achieve suppression. The suppression effectors of choice in the invention have been hammerhead ribozymes with antisense flanks to define sequence specificity. Hammerhead ribozymes require NUX cleavage sites in open loop structures of RNA. Notably, other suppression effectors could be utilised in the invention and may lead to a more flexible choice of target sequences for suppression. Transcripts expressed from all four genes have been significantly attacked in vitro using suppression effectors directed towards target

cleavage sites. In all four examples the ribozymes directed to cleavage sites were successful in cleaving target RNAs in the predicted manner. Antisense complementary to sequences surrounding the cleavage sites was used successfully

to elicit binding and cleavage of target RNAs in a sequence specific manner. Additionally, transcripts from replacement genes, modified using the degeneracy

of the genetic code so that they code for wild type protein, were protected fully from cleavage by ribozymes.

_____The utility of an individual ribozyme designed to target an NUX site in an open loop structure of transcripts from a gene will depend in part on the robust nature of the RNA open loop structure when various deleterious mutations are also present in the transcript. To evaluate this, we analysed RNAPlotFold data for six different adRP causing mutations in the rhodopsin gene. For each of these, the large RNA open loop structure which is targeted by Rz10 was predicted

to be maintained in the mutant transcripts (Table 2A). This is clearly demonstrated in example 1B (Figure 34) using a Pro23Leu rhodopsin mutation. Rz10 clearly cleaves the mutant transcript effectively *in vitro*. The Pro23Leu mutation creates a ribozyme cleavage site and can be cleaved *in vitro* by Rz20 a ribozyme specifically

|
|
| targeting this site -- however this is not the case for
| many mutations. In contrast we have shown that the Rz10
| ribozyme cleavage site is available for different mutant
| rhodopsins and could potentially be used to suppress
5 | multiple mutations using a suppression and replacement
| approach.

|
| _____ In some cases lowering RNA levels may lead to a
| parallel lowering of protein levels however this may not
10 | always be the case. In some situations mechanisms
|
| may prevent a significant decrease in protein levels
| despite a substantial decrease in levels of RNA. However
| in many instances suppression at the RNA level has been
| shown to be effective (see prior art). In some cases it
15 | is thought that ribozymes elicit suppression not only by
| cleavage of RNA but also by an antisense effect due to
| the antisense arms of the ribozyme surrounding the
| catalytic core.

|
| _____
20 | In all examples provided ribozymes were designed to
| cleave at specific target sites. Target sites for four
| of the ribozymes utilised were chosen in open loop
|
| structures in the coding regions of transcripts from
| three retinal genes (human and mouse rhodopsin and human
25 | ~~peripherin~~peripherin). In all cases sequence specific

cleavage was obtained at the target cleavage sites (Figs 1--7). Target sites were chosen in open loop structures to optimise cleavage. Additionally target sites were chosen such that they could be obliterated by single nucleotide changes at third base wobble positions and therefore would code for the same amino acid (Table 2B). In turn this enabled the generation of replacement genes with single nucleotide alterations which code for wild type protein. In all cases tested transcripts from replacement genes were protected from cleavage by ribozymes. Further modifications could be made to replacement genes in wobble positions, for example, to limit the binding ability of the antisense arms flanking the ribozyme catalytic core. The examples provided for rhodopsin and peripherin involve suppression of expression of both disease and wild type alleles of a retinal gene and restoration of the wild type protein using a replacement gene. However, there may be situations where single alleles can be targeted specifically or partially specifically (PCT/GB97/00574).

____ In one example, human collagen 1A2, Rz907 was used to target a naturally occurring polymorphic site at amino acid 187, (GGA (glycine) ---> GGT (glycine), located in an open loop structure from the predicted 2--D conformations of the transcript (Figure #9, Table 2B). The ribozyme Rz907 cleaved transcripts containing the GGT

sequence but transcripts with GGA were protected from cleavage. Transcripts from both alleles of individuals homozygous for the GGT polymorphism could be cleaved by Rz907 whereas in the case of heterozygotes cleavage could
5 be directed to single alleles (in particular to alleles containing deleterious mutations PCT/GB97/00574). In both situations replacement genes could have the sequence GGA and therefore would be protected from cleavage by Rz907. The presence of many such naturally occurring
10 silent polymorphisms highlights that replacement genes could be modified in a similar fashion in wobble positions and should produce in most cases functional wild type protein. Multiple modifications could be made to replacement genes at wobble positions
15 which would augment protection from suppression effectors. For example, in situations where antisense nucleic acids were used for suppression, transcripts from replacement genes with multiple modifications at third base positions would be protected partially or
20 completely from antisense binding.

____ In all four examples provided transcripts from cDNA clones were cleaved *in vitro* in a sequence specific manner at ribozyme cleavage sites. Additionally one base
25 of the ribozyme cleavage site occurs at a wobble position

and moreover can be altered so as to eliminate the
cleavage site. Ribozyme cleavage sites in the
examples given were destroyed by changing nucleotide
sequences so that the consensus sequence for ribozyme
5 | cleavage sites was broken. However it may be
that in some cases the cleavage site could be destroyed
by altering the nucleotide sequence in a manner that
alters the 2nd structure of the RNA and destroys the
open loop structure targeted by the ribozyme. cDNAs or
10 | DNA fragments with altered sequences in the regions
targeted by ribozymes were generated. RNAs expressed
from these cDNAs or DNA fragments were protected entirely
from cleavage due to the absence of the ribozyme cleavage
site for each of the ribozymes tested. Of particular
15 | interest is the fact that a single nucleotide alteration
can obliterate a ribozyme target site, thereby preventing
RNA cleavage. Although ribozymes have been used in the
demonstration of the invention, other suppression
effectors could be used to achieve gene silencing. Again
20 | replacement genes with altered sequences (at third base
wobble positions) could be generated so that they are
protected partially or completely from gene silencing and
provide the wild type (or beneficial) gene product.

_____As highlighted before in the text, using the invention the same method of suppression (targeting coding sequences of a gene) and where necessary gene replacement (using a replacement gene with a sequence modified in third base positions to restore gene expression) may be used as a therapeutic approach for many different mutations within a given gene. Given the continuing elucidation of the molecular pathogenesis of dominant and polygenic diseases the number of targets for this invention is rapidly increasing.

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We claim:



|

|

CLAIMS

1 1. A strategy for suppressing or partially suppressing
2 an endogenous gene and replacing the suppressed gene
3 sequence with a nucleic acid sequence which differs
4 from the endogenous gene and wherein the suppressing
5 agent(s) comprises at least one suppressor from the
6 group comprising antisense nucleic acid, peptide
7 nucleic acids, DNA capable of forming triple helix or
8 ribozymes targeted to the endogenous gene or gene
9 transcripts and wherein the replacement nucleic acid
10 sequence encodes at least part of a gene product and
11 is not suppressed by suppression agent(s) or is
12 suppressed less efficiently by suppression agent(s)
13 and wherein the replacement nucleic acid sequence
14 comprises amino acid codons which encode at least
15 part of the gene product, and have modifications at
16 wobble site(s) such that replacement nucleic acids
17 still code for the wild type or equivalent amino
18 acids.

1

2

3 2. A medicament comprising either one or both of a gene
4 suppressing agent and a nucleic acid encoding at

5 least part of a replacement gene product for use in a
6 strategy as claimed in Claim 1.

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3 3. A medicament comprising a nucleic acid sequence
4 encoding at least part of a gene product wherein the
5 sequence differs from the endogenous gene in wobble
6 sites.

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3 4. A strategy for suppressing or partially suppressing
4 an endogenous gene and introducing a replacement gene
5 said strategy comprising the steps of:

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7 a. providing suppression nucleic acids or other
8 suppression effector(s) able to recognise, bind
9 or cleave an endogenous gene, gene transcript(s)
10 or gene product to be suppressed and

11 b. providing genomic DNA or cDNA (complete or
12 partial) encoding a replacement gene wherein the
13 suppression nucleic acids are unable to
14 recognise, bind or cleave or able to recognise,
15 bind or cleave less efficiently equivalent

16 regions in the genomic DNA or cDNA to prevent
17 suppression of the replacement gene wherein the
18 coding sequence of replacement nucleic acids has
19 been altered to prevent or reduce efficiency of
20 suppression and wherein replacement nucleic
21 acids have modifications in one or more wobble
22 sites such that replacement nucleic acids still
23 code for the wild type or equivalent amino
24 acids.

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3 5. The use of a strategy as claimed in any of the
4 preceding Claims in the preparation of a medicament
5 for the treatment of an autosomal dominant disease
6 caused by an endogenous target gene wherein the
7 disease is caused by different mutations in the same
8 gene in different patients.

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3 6. The use of:

4 a. a vector or vectors containing suppression
5 effector(s), said suppression effector(s) being
6 able to recognise, bind or cleave coding
7 sequences of a target endogenous gene and

8 b. vector(s) containing replacement nucleic acids
9 in the form of genomic DNA, cDNA or RNA, which
10 contain altered wobble sites such that
11 replacement nucleic acids cannot be recognised,
12 bound or cleaved by suppressor(s) or are
13 recognised, bound or cleaved less efficiently by
14 suppressor(s) which are targeted towards coding
15 sequence of the endogenous gene and which
16 provide the wild type gene product and wherein
17 the difference between said endogenous gene and
18 the replacement gene still enables the
19 expression of the replacement gene,

20
21 in the preparation of a medicament for the treatment
22 of an autosomal dominant disease caused by the
23 endogenous gene wherein the disease is caused by
24 different mutations in the same gene in different
25 patients.

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3 7. A use as claimed in Claims 5 or 6 wherein the disease
4 is a polygenic disorder.

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3 8. A use as claimed in Claim 6 or 7 wherein
4 suppressor(s) or replacement gene(s) are administered
5 alone or in vector(s) chosen from DNA plasmid
6 vectors, RNA or DNA viral vectors.

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3 9. A use as claimed in Claim 8 wherein the suppressor(s)
4 or replacement gene(s) are combined with lipids,
5 polymers or other derivatives.

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3 10. A kit for use in the treatment of an autosomal
4 dominant or polygenic disease caused by mutation(s)
5 in a target endogenous gene, the kit comprising at
6 least one suppression effector able to recognise,
7 bind or cleave coding sequence(s) of the endogenous
8 gene to be suppressed and at least one replacement
9 gene to replace the endogenous gene having
10 modifications to wobble sites such that the
11 replacement gene cannot be recognised, bound or
12 cleaved or can be recognised, bound or cleaved less
13 efficiently by suppressor(s) targeted to coding
14 sequence(s) of the endogenous gene, said replacement
15 nucleic acid sequence providing the wild type gene
16 product, and wherein the difference between said wild

17 type target gene and the replacement gene still
18 enables expression of the replacement gene.

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3 11. A use as claimed as in Claims 1 to 10 wherein the
4 replacement gene is altered from the wild type gene
5 and provides a beneficial effect when compared to the
6 wild type gene.

ABSTRACT

~~"Genetic Suppression and Replacement"~~

A strategy for suppressing specifically or partially specifically an endogenous gene and introducing a replacement gene, said strategy comprising the steps of:

1. providing suppressing nucleic acids or other suppression effectors able to bind to an endogenous gene, gene transcript or gene product to be suppressed and
2. providing genomic DNA or cDNA (complete or partial) encoding a replacement gene wherein the suppressing nucleic acids are unable to bind to equivalent regions in the genomic DNA or cDNA to prevent expression of the replacement gene.

The replacement nucleic acids have modifications in one or more third base (wobble) positions such that replacement

nucleic acids still code for the wild type or equivalent
amino acids.

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